Published in final edited form as:

Brain Res. 2011 March 16; 1379: 2–10. doi:10.1016/j.brainres.2010.11.090.

Estrogen Protection Against Mitochondrial Toxin-Induced Cell Death In Hippocampal Neurons: Antagonism by Progesterone

Jia Yao, Shuhua Chen, Enrique Cadenas, and Roberta Diaz Brinton

Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, CA 90033

Abstract

Previously we demonstrated that mitochondrial dysfunction plays a critical role in the pathogenesis of Alzheimer's disease. Further, we have shown that the neuroprotective effects of 17 β -estradiol (E2) are dependent upon mitochondrial function. In the current study, we sought to identify mitochondrial sites of E2 action that mediate neuroprotection by assessing the efficacy of E2 to protect neurons against inhibitors of mitochondrial respiration which target specific complexes within the respiratory chain. Subsequently, the impact of progesterone (P4) on E2-induced prevention against mitochondrial toxins was investigated. Mitochondrial inhibitors, rotenone, 3-NPA, antimycin, KCN, and oligomycin, exhibited concentration dependent toxicity in primary hippocampal neurons. The concentration inducing 30% cell death (LD30) was selected for analyses assessing the neuroprotective efficacy of ovarian hormones (E2 and P4). Pretreatment of hippocampal neurons with E2 significantly protected against 3-NPA (7.5mM) and antimycin (125 μ M) induced cell death and was moderately neuroprotective against rotenone (3 μ M). E2 was ineffective against KCN and oligomycin-induced cell death. Pretreatment with P4 was without effect against these mitochondrial inhibitors. Co-administration of P4 with E2 abolished E2 induced neuroprotection against 3-NPA and antimycin. Additional metabolic analyses indicated that E2 and P4 separately increased mitochondrial respiratory capacity whereas the co-administration of E2 and P4 resulted in diminished mitochondrial respiration. These findings indicate that E2 protects against mitochondrial toxins that target Complexes I, II and III whereas P4 was without effect. The data also predict that continuous combined co-administration of estrogen and progesterone common to many hormone therapy regimens is unlikely to prevent the deficits in mitochondrial function.

Keywords

estrogen; progesterone; mitochondria; oxidative stress; Alzheimer's disease

1. Introduction

Overall, basic science analyses using both *in vitro* and *in vivo* models indicate that estrogen, typically 17 β -estradiol (E2) but also conjugated equine estrogens, protect neurons against brain insults associated with Alzheimer's disease (Brinton, 2008a; Chen et al., 2006).

© 2010 Elsevier B.V. All rights reserved.

Corresponding Author: Roberta Diaz Brinton, Ph.D., University of Southern California, School of Pharmacy, 1985 Zonal Avenue, PSC-502, Los Angeles, CA 90033, Voice: 323-442-1436, FAX: 323-442-1740, rbrinton@usc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Pretreatment with E₂ can protect against a wide range of toxic insults including free radical generators (Behl et al., 1995; Green et al., 2001), excitotoxicity, β amyloid-induced toxicity (Chen et al., 2006) and ischemia (Dubal et al., 1998; Green et al., 2001). Moreover, estrogen has been demonstrated to activate biochemical, genomic, cellular and behavioral mechanisms of memory (Brinton, 2009; McEwen, 2002; Simpkins et al., 2009; Singh et al., 2006; Wise et al., 2001; Woolley, 1999). We have previously shown that many of the neuroprotective mechanisms of estrogen converge upon mitochondria. We have demonstrated that E₂ pretreatment prevents mitochondrial dysfunction by promoting the maintenance of mitochondrial Ca²⁺ homeostasis (Nilsen and Brinton, 2002). Further, E₂ increases the oxidative capacity and efficiency of brain mitochondria (Irwin et al., 2008; Nilsen et al., 2007). This increased oxidative efficiency by increased expression of subunits of both Complex IV and V is correlated with increased Manganese Superoxide Dismutase (MnSOD) and peroxiredoxin expression and reduced lipid peroxidation. Consistent with these findings, E₂-treatment increased the activity of the key glycolytic enzymes hexokinase, phosphofructokinase and phosphoglycerate kinase in rodent brain (Kostanyan and Nazaryan, 1992).

Previous studies indicated that mitochondria are a key target of estrogen action in the brain (Brinton, 2008b; Nilsen and Diaz Brinton, 2003; Simpkins et al., 2009; Singh et al., 2006; Yao et al., 2009; Yao et al., 2010). Further, individually both E₂ and progesterone (P₄) can promote mitochondrial function with E₂ promoting mitochondrial function and antioxidant pathway whereas P₄ promotes mitochondria function with variable regulation of antioxidant enzymes (Irwin et al., 2008; Nilsen and Brinton, 2002).

In the current study, we sought to determine specific sites of E₂ and P₄ regulation of the oxidative phosphorylation machinery within the mitochondrial electron transport chain (mETC) using mitochondrial inhibitors specific for each mETC complex. We further assessed the impact of E₂+P₄ co-administration on protection against mitochondrial toxins as well as mitochondrial bioenergetic function. Findings from this study demonstrated that E₂ induced significant protection against specific mitochondrial inhibitors. In contrast, P₄ exhibited no protection against mitochondrial inhibitors and the co-administration of P₄ with E₂ abolished E₂ induced neuroprotection. Bioenergetically, the co-administration of E₂ and P₄ diminished the up-regulation of mitochondrial respiration relative to E₂ or P₄ treatment alone. From a clinical perspective, these data suggest that continuous combined co-administration of estrogen and progesterone common to many hormone therapy regimens is unlikely to sustain mitochondrial function and protect mitochondria from age- and neurodegenerative related insults.

2. Results

2.1 Concentration dependent toxicity of different mitochondrial inhibitors

Embryonic day 18 (E18) primary hippocampal neurons were cultured for 10 days prior to treatment of increasing concentrations of mitochondrial inhibitors that target different sites within the mETC (Fig. 1A). Rotenone binds and inhibits complex I. 3-NPA is a specific inhibitor for succinate dehydrogenase (SDH, complex II). Antimycin inhibits complex III whereas KCN inhibits complex IV, cytochrome c oxidase. Oligomycin is an ATP synthase inhibitor and inhibits the synthesis from ADP to ATP. Cell viability was measured 24 hours after exposure to mitochondrial inhibitors. All mitochondrial inhibitors exhibited a concentration dependent toxicity (Fig. 1B – 1F). Cell death induced by mitochondrial inhibitors is likely due to energy inhibition coupled with increased oxidative stress. The inhibition of the complexes in the mETC not only inhibits electron flow through the mETC, hence decreasing ATP production, but also induces increased free radical generation and

oxidative stress. We chose the toxin concentration that induced approximately 30% cell death to assess E2/P4 induced neuroprotection.

2.2 E2 induced neuroprotection against mitochondrial toxins

Upon determining the toxicity profile of different mitochondrial inhibitors, we continued to investigate the therapeutic efficacy of E2 induced neuroprotection against these toxins. In this study, we selected the optimal toxin concentration that could induce about 30% cell death to ensure sufficient cell death without the activation of the irreversible cell death at high toxin concentrations. Pretreatment of E2 at different concentrations induced significant neuroprotection against 3-NPA (7.5mM) antimycin (125 μ M), and rotenone (3 μ M), whereas E2 showed no protection or even adverse impact against other inhibitors, such as KCN and Oligomycin (Fig. 2).

2.3 P4 exhibited no neuroprotection against mitochondrial inhibitors

Aside from estrogen, other ovarian hormones have also been demonstrated protect neurons from a variety of insults. We have previously shown that progesterone (P4) also regulates mitochondrial function *in vivo* by promoting mitochondrial respiration with less regulation of the antioxidant system (Irwin et al., 2008). In the current study, we investigated the neuroprotective efficacy of P4 against mitochondrial toxins. Unlike E2, pretreatment of P4 exhibited no neuroprotection against mitochondrial inhibitors (Fig. 3).

2.4 Co-administration of P4 abolished E2 induced neuroprotection against mitochondrial inhibitors

Previous studies indicated an antagonistic interaction between E2 and P4 that abolished the neuroprotective benefits of both hormones (Carroll et al., 2007; Carroll et al., 2008; Irwin et al., 2008). Based on these previous findings and the common use of continuous combined hormone therapy, we investigated the impact of co-administration of P4 and E2 on their neuroprotective efficacy against mitochondrial toxins. Co-administration of P4, particularly at concentrations equal to or higher than E2, significantly abolished E2 induced neuroprotection against 3-NPA and Antimycin (Fig. 4).

2.5 Co-administration of P4 abolished E2/P4 induced enhancement of mitochondrial function

Mitochondrial toxins can directly inhibit mitochondrial oxidative phosphorylation required for energy production and simultaneously increase oxidative stress associated by inhibiting electron flow through the mETC. The neuroprotective benefits of E2 could be partially attributed to E2 induced enhancement of mitochondrial function (Henderson, 2010). To further investigate E2 and P4 regulation of mitochondrial bioenergetic function, we conducted *in vitro* metabolic assays to determine the impact of E2, P4, and the co-administration of E2+P4 on both mitochondrial respiration and anaerobic glycolysis. Both E2 (10ng/ml) and P4 (100ng/mL) increased the maximal mitochondrial respiratory capacity as indicated by the OCR (oxygen consumption rate) value. However, co-administration of E2+P4 (10ng/ml E2+100ng/ml P4) exhibited diminished effect on mitochondrial respiration relative to E2 or P4 treatment alone (Fig. 5A). These findings are consistent with findings from our previous *in vivo* study that short term E2 or P4 treatment enhanced brain mitochondrial function as evidenced by increased respiratory control ratios whereas co-administration of E2+P4 did not promote brain mitochondrial respiration. In addition to aerobic respiration, we also assessed *in vitro* E2/P4 regulation of anaerobic glycolysis as indicated by ECAR (extra-cellular acidification rate) value. Neither E2 or P4 alone nor co-administration of E2+P4 altered the anaerobic glycolytic capacity of the neurons (Fig. 5B), suggesting that the potentiation of E2 or P4 alone on mitochondrial respiration mainly

results from a direct increase in mitochondrial oxidative phosphorylation capacity by increasing expression of mETC complexes rather than a secondary enhancement due to increased substrate availability from enhanced glycolytic activity.

3. Discussion

In the current study, we demonstrated the differential profiles of E2 and P4 induced neuroprotection against mitochondrial inhibitors. E2 pretreatment exhibited neuroprotection against specific mitochondrial inhibitors whereas P4 pretreatment had little benefits against these inhibitors. Further, P4 co-administration abolished E2 induced neuroprotection. Results presented here expand our current understanding of the basic mechanisms of ovarian hormone induced neuroprotection. From a translational perspective, findings from this study contribute to designing an optimal treatment paradigm for combined hormone therapy.

3.1 Mitochondrial mechanisms of E2 induced neuroprotection

Mitochondrial function is a key regulator of aging and age-related neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease (Beal, 2005; Lin and Beal, 2006). We have previously demonstrated that the neuroprotective mechanisms of estrogen converge upon mitochondria (Brinton, 2008a; Brinton, 2008b; Irwin et al., 2008; Nilsen et al., 2007). The mechanisms of estrogen regulating mitochondrial function are multifaceted, including both the activation of classic estrogen receptor (ER) pathways, and the activation of membrane ERs and the downstream CREB/ERK pathway (Brinton, 2008a; Brinton, 2009; Mannella and Brinton, 2006; Singh et al., 2006). In the current study, we expanded the investigation by determining the sites of estrogen action within the mETC in primary neuronal cultures. Using mitochondrial inhibitors/toxins that are specific for each complex within the mETC, we demonstrated that E2 pretreatment significantly protected against complex I inhibitor, rotenone, complex II inhibitor 3-NPA, complex III inhibitor antimycin. In contrast, E2 pretreatment exhibited no neuroprotection against complex IV inhibitor, KCN or complex V inhibitor, Oligomycin.

E2 has long been demonstrated to up-regulate anti-apoptotic proteins such as Bcl-2 and Bcl-xL (Irwin et al., 2008; Nilsen et al., 2006). The anti-apoptotic property of E2 should induce a uniform protection against all mitochondrial toxins. However, in the current study E2 pretreatment selectively protected neurons against a subset of mitochondrial inhibitors, thus indicating a site-specific mechanism of neuroprotection rather than a universal neuroprotective effect. The neuroprotective efficacy of E2 against mitochondrial inhibitors can likely be attributed to both sustaining energy production and preventing oxidative stress. Estrogen has long been demonstrated to up-regulate mitochondrial proteins, including mETC complex subunits (Bieber and Cohen, 2001; Brinton, 2008a; Simpkins et al., 2009). In addition, estrogen has also been demonstrated to promote anti-oxidant defense system by increasing the expression and/or activity of import enzymes such as MnSOD and peroxiredoxin V (Prdx V) (Borras et al., 2010). In this study, E2 pretreatment failed to protect neurons against KCN toxicity, despite E2 induced up-regulation of complex IV subunits (Nilsen and Diaz Brinton, 2003; Nilsen et al., 2006). Considering that complex IV is the terminal enzyme of electron flow where O_2 is reduced to H_2O , inhibition of complex IV by KCN not only induces a severe insult on both energy production and oxygen consumption but also leads to an increase in oxidative stress. Up-regulation in a subset of complex IV subunits by E2 is unlikely to offset KCN induced dysfunction of complex IV.

In addition, E2 also failed to protect neurons from oligomycin toxicity. We have previously shown that E2 increases the expression of catalytic α subunit (F1 α) of complex V (ATP synthase) (Irwin et al., 2008; Nilsen et al., 2007). However, oligomycin inhibits ATP synthesis by blocking the proton channel (F0 complex). Up-regulation of F1 expression by

E2 cannot alleviate the inhibition of the proton channel and therefore exhibited no protection against oligomycin.

3.2 Mechanisms of P4 induced neuroprotection: different profile from E2

Emerging evidence indicates that progesterone has multiple actions in the central nervous system to regulate cognition, mood, neurogenesis and neuro-regeneration (Barron et al., 2006; Brinton et al., 2008; Gibson et al., 2007; Schumacher et al., 2007; Schumacher et al., 2008). Metabolic analyses from this study demonstrated that P4, promoted mitochondrial respiration *in vitro* as did E2. These findings are consistent with previous *in vivo* findings that P4 potentiates brain mitochondrial function (Irwin et al., 2008; Singh, 2006). In contrast, P4 did not protect against electron transport chain inhibitors. Compared to E2, P4 had limited efficacy to prevent oxidative damage (Goodman et al., 1996). Previously we demonstrated that P4 did not increase the expression of antioxidant enzyme Prdx V, and was much less efficacious in reducing lipid peroxidation relative to E2 (Irwin et al., 2008). The toxicity of mETC inhibitors is inextricably linked with increased oxidative stress, which significantly adds to the neurotoxicity of these compounds. E2 protects neurons by simultaneously potentiating mitochondrial bioenergetic capacity and preventing or reducing the oxidative stress induced by mETC inhibitors. P4, despite its enhancement of mitochondrial respiration, is less efficacious in offsetting the increase in oxidative insults associated with increased oxidative phosphorylation (Irwin et al., 2008).

3.3 Antagonistic mechanisms between E2 and P4

In addition to investigating the impact of E2 or P4 treatment alone, we assessed the impact of E2+P4 co-administration on both neuroprotection against mitochondrial inhibitors and mitochondrial bioenergetic capacity. Data demonstrated that P4, especially at concentrations equal or higher than E2, significantly abolished E2 induced protection against mitochondrial inhibitors. Similarly, metabolic analyses indicated that the co-administration of P4 with E2 induced much less potentiation of mitochondrial bioenergetic capacity, whereas when treated alone, both P4 and E2 enhanced mitochondrial respiration. These findings are in agreement with previous findings that the co-administration of progesterone and estrogen results in diminished benefits of the individual steroids when administered alone (Brinton et al., 2008; Carroll et al., 2007; Carroll et al., 2008; Hoffman et al., 2006; Nilsen and Brinton, 2002; Rosario et al., 2006). Although the antagonistic mechanism of progesterone on estrogen-inducible responses is yet to be fully understood, it has been suggested that progesterone attenuates the neuroprotective effects of estrogen by down-regulating ER α and ER β , *in vitro* (Jayaraman and Pike, 2009). Both ER α and ER β have been demonstrated to mediate estrogen induced neuroprotection (Cordey and Pike, 2005; Mannella and Brinton, 2006; Singh et al., 2006; Yager and Chen, 2007; Zhao et al., 2004). Decrease in ER expression by progesterone could partially account for the diminished benefits of E2+P4 co-administration.

3.4 Clinical Implications

From a clinical perspective, progesterone or clinical progestins are widely used in conjunction with different types of estrogens in both oral contraceptives and hormone therapies. Previous studies have demonstrated that progesterone antagonizes estrogen induced protection against neurotoxic insults (Carroll et al., 2008; Irwin et al., 2008; Nilsen and Brinton, 2002). In the current study, the combination of E2+P4 was significantly less efficacious in promoting mitochondrial respiration than either E2 alone or P4 alone. Further, simultaneous exposure to E2+P4 abolished E2 induced protection against mETC inhibitors. Together, these findings indicate that continuous combined co-administration of estrogen and progesterone common to many hormone therapy regimens results in diminished protection against mETC inhibitors and diminution of mitochondrial function. These findings

have potential clinical relevance for long term use of continuous combined hormone therapies in post-menopausal women and their risk of age-associated decline in brain mitochondrial function and neurodegenerative diseases such as Alzheimer's.

4. Experimental Procedure

4.1 Tissue Culture

The use of animals for the study was conducted following National Institutes of Health guidelines on use of laboratory animals and was approved by the Institutional Animal Care and Use Committee at the University of Southern California. Minimal numbers (n=9) of animals have been used to complete the study. Embryonic day 18 (E18) primary hippocampal neurons derived from female Sprague-Dawley pregnant rats (Harlan, Indianapolis, IN) were cultured as previously described and generated cultures 98% neuronal in phenotype (Nilsen et al., 2006). Briefly, embryonic rat hippocampi were dissociated by passage through fire-polish constricted Pasteur pipettes. Neurons plated on polyethylenimine precoated 96 well plates or 60 mm petri-dishes were grown in Neurobasal Medium +B27 supplement at 37°C in humidified 5% CO₂ atmosphere for 10–12 days prior to experimentation.

4.2 Steroid Treatment and Neuroprotection Assay

To determine the toxicity of mitochondrial inhibitors, neurons were cultured 12 days prior to the exposure to either vehicle or individual mitochondrial toxins at different concentrations. Briefly, rotenone concentrations range from 5μM to 15μM; 3-NPA concentrations range from 2.5mM to 15mM; Antimycin concentrations range from 62.5μM to 150μM; KCN concentrations range from 10mM to 30mM; oligomycin concentrations range from 1μM to 5μM. 24 hours later, cell viability was measured and the toxin concentration that induced about 25% to 30% of cell death was chosen to measure estrogen and progesterone induced neuroprotection against these toxins. Briefly, neurons were cultured for 11 days and treated with ascending concentrations of E2 or P4 (0.1ng/ml, 1ng/ml, 10ng/ml, 100ng/ml, and 1000ng/ml) 24 hours prior to the exposure of toxins. After another 24 hour exposure to the toxins (10μM rotenone, 7.5mM 3-NPA, 125μM antimycin, 20mM KCN, and 3μM oligomycin, respectively), cell viability was measured. To determine whether the co-administration of P4 antagonizes E2 induced neuroprotection, neurons were cultured for 11 days and treated with 10ng/ml of E2 with ascending concentrations of P4 (0.1ng/ml, 1ng/ml, 10ng/ml, 100ng/ml, and 1000ng/ml) 24 hours prior to the exposure to 10mM 3-NPA or 125μM Antimycin. Cell Viability was measured 24 hours after toxin treatment.

4.3 Cell Viability Assays

Neuronal viability was assessed by Calcein AM staining monitoring fluorescence (Ex: 485 nm/Em: 530 nm) on a fluorescent plate reader. Each experiment consisted of 8 wells per condition. Means were normalized to the control values for comparison across experiments. Data is presented as means ± S.E.M. from at least 3 independent experiments. Neuroprotective efficacy was defined as $(\text{Via}_{\text{E2}} - \text{Via}_{\text{tox}}) / (\text{Via}_{\text{veh}} - \text{Via}_{\text{tox}}) * 100\%$.

4.4 Seahorse XF-24 Metabolic Flux Analysis

To investigate the impact of E2 and P4 co-administration of cellular metabolic flux activity, primary hippocampal neurons from day 18 (E18) embryos of female Sprague-Dawley rats were cultured on Seahorse XF-24 plates at a density of 50,000 cells/well. Neurons were grown in Neurobasal Medium +B27 supplement for 10 days prior to exposure of E2 (10ng/ml), P4 (100ng/ml), and E2 (10ng/mL)+P4 (100ng/mL), respectively. Metabolic flux activity was measured 24 hours later. On the day of metabolic flux analysis, cells were

changed to unbuffered DMEM (DMEM Base medium supplemented with 25mM glucose, 1mM sodium pyruvate, 31mM NaCl, 2mM GlutaMax; pH 7.4) and incubated at 37°C in a non-CO₂ incubator for 1 h. All medium and injection reagents were adjusted to pH 7.4 on the day of assay. Four baseline measurements of OCR and ECAR were taken before sequential injection of mitochondrial inhibitors. Three readings were taken following each addition of mitochondrial inhibitor prior to injection of the subsequent inhibitors. The mitochondrial inhibitors used were oligomycin (1 µM), FCCP (1 µM), and rotenone (1 µM). OCR and ECAR were automatically calculated and recorded by the Seahorse XF-24 software. After the assays, plates were saved and protein readings were measured for each well in order to confirm equal cell numbers per well. The percentage of change compared to the basal rates was calculated as the value of change divided by the average value of baseline readings.

4.5 Statistics

Statistically significant differences between groups were determined by an ANOVA followed by a Newman-Keuls post-hoc analysis.

Acknowledgments

This study was supported by National Institute on Aging Grant 2R01AG032236 (to RDB), National Institute on Aging Grant 5P01AG026572 (to RDB) and the Kenneth T. and Eileen L. Norris Foundation (to RDB).

Abbreviations

E2	17β-estradiol
P4	Progesterone
mETC	mitochondrial Electron Transport Chain
OCR	Oxygen Consumption Rate
ECAR	Extra-cellular Acidification Rate

References

- Barron AM, Fuller SJ, Verdile G, Martins RN. Reproductive hormones modulate oxidative stress in Alzheimer's disease. *Antioxid Redox Signal*. 2006; 8:2047–59. [PubMed: 17034349]
- Beal MF. Mitochondria take center stage in aging and neurodegeneration. *Ann Neurol*. 2005; 58:495–505. [PubMed: 16178023]
- Behl C, Widmann M, Trapp T, Holsboer F. 17-beta estradiol protects neurons from oxidative stress-induced cell death in vitro. *Biochem Biophys Res Commun*. 1995; 216:473–82. [PubMed: 7488136]
- Bieber EJ, Cohen DP. Estrogens and hormone replacement therapy: is there a role in the preservation of cognitive function? *Int J Fertil Womens Med*. 2001; 46:206–9. [PubMed: 11563830]
- Borras C, Gambini J, Lopez-Grueso R, Pallardo FV, Vina J. Direct antioxidant and protective effect of estradiol on isolated mitochondria. *Biochim Biophys Acta*. 2010; 1802:205–11. [PubMed: 19751829]
- Brinton RD. The healthy cell bias of estrogen action: mitochondrial bioenergetics and neurological implications. *Trends Neurosci*. 2008a; 31:529–37. [PubMed: 18774188]
- Brinton RD. Estrogen regulation of glucose metabolism and mitochondrial function: therapeutic implications for prevention of Alzheimer's disease. *Adv Drug Deliv Rev*. 2008b; 60:1504–11. [PubMed: 18647624]

- Brinton RD, Thompson RF, Foy MR, Baudry M, Wang J, Finch CE, Morgan TE, Pike CJ, Mack WJ, Stanczyk FZ, Nilsen J. Progesterone receptors: form and function in brain. *Front Neuroendocrinol.* 2008; 29:313–39. [PubMed: 18374402]
- Brinton RD. Estrogen-induced plasticity from cells to circuits: predictions for cognitive function. *Trends Pharmacol Sci.* 2009; 30:212–22. [PubMed: 19299024]
- Carroll JC, Rosario ER, Chang L, Stanczyk FZ, Oddo S, LaFerla FM, Pike CJ. Progesterone and estrogen regulate Alzheimer-like neuropathology in female 3xTg-AD mice. *J Neurosci.* 2007; 27:13357–65. [PubMed: 18045930]
- Carroll JC, Rosario ER, Pike CJ. Progesterone blocks estrogen neuroprotection from kainate in middle-aged female rats. *Neurosci Lett.* 2008; 445:229–32. [PubMed: 18790007]
- Chen S, Nilsen J, Brinton RD. Dose and temporal pattern of estrogen exposure determines neuroprotective outcome in hippocampal neurons: therapeutic implications. *Endocrinology.* 2006; 147:5303–13. [PubMed: 16916950]
- Cordey M, Pike CJ. Neuroprotective properties of selective estrogen receptor agonists in cultured neurons. *Brain Res.* 2005; 1045:217–23. [PubMed: 15910780]
- Dubal DB, Kashon ML, Pettigrew LC, Ren JM, Finklestein SP, Rau SW, Wise PM. Estradiol protects against ischemic injury. *J Cereb Blood Flow Metab.* 1998; 18:1253–8. [PubMed: 9809515]
- Gibson CL, Gray LJ, Bath PM, Murphy SP. Progesterone for the treatment of experimental brain injury; a systematic review. *Brain.* 2007
- Goodman Y, Bruce AJ, Cheng B, Mattson MP. Estrogens attenuate and corticosterone exacerbates excitotoxicity, oxidative injury, and amyloid beta-peptide toxicity in hippocampal neurons. *J Neurochem.* 1996; 66:1836–44. [PubMed: 8780008]
- Green PS, Yang SH, Nilsson KR, Kumar AS, Covey DF, Simpkins JW. The nonfeminizing enantiomer of 17beta-estradiol exerts protective effects in neuronal cultures and a rat model of cerebral ischemia. *Endocrinology.* 2001; 142:400–6. [PubMed: 11145603]
- Henderson, VaBRD. Menopause and Mitochondria: Windows into Estrogen Effects on Alzheimer's Disease Risk and Therapy: Progress in Brain Research Neuroendocrinology. 2010 In press.
- Hoffman GE, Merchenthaler I, Zup SL. Neuroprotection by ovarian hormones in animal models of neurological disease. *Endocrine.* 2006; 29:217–31. [PubMed: 16785598]
- Irwin RW, Yao J, Hamilton RT, Cadenas E, Brinton RD, Nilsen J. Progesterone and estrogen regulate oxidative metabolism in brain mitochondria. *Endocrinology.* 2008; 149:3167–75. [PubMed: 18292191]
- Jayaraman A, Pike CJ. Progesterone attenuates oestrogen neuroprotection via downregulation of oestrogen receptor expression in cultured neurones. *J Neuroendocrinol.* 2009; 21:77–81. [PubMed: 19094096]
- Kostanyan A, Nazaryan K. Rat brain glycolysis regulation by estradiol-17 beta. *Biochim Biophys Acta.* 1992; 1133:301–6. [PubMed: 1531302]
- Lin MT, Beal MF. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature.* 2006; 443:787–95. [PubMed: 17051205]
- Mannella P, Brinton RD. Estrogen receptor protein interaction with phosphatidylinositol 3-kinase leads to activation of phosphorylated Akt and extracellular signal-regulated kinase 1/2 in the same population of cortical neurons: a unified mechanism of estrogen action. *J Neurosci.* 2006; 26:9439–47. [PubMed: 16971528]
- McEwen B. Estrogen actions throughout the brain. *Recent Prog Horm Res.* 2002; 57:357–84. [PubMed: 12017552]
- Nilsen J, Brinton RD. Impact of progestins on estradiol potentiation of the glutamate calcium response. *Neuroreport.* 2002; 13:825–30. [PubMed: 11997695]
- Nilsen J, Diaz Brinton R. Mechanism of estrogen-mediated neuroprotection: regulation of mitochondrial calcium and Bcl-2 expression. *Proc Natl Acad Sci U S A.* 2003; 100:2842–7. [PubMed: 12604781]
- Nilsen J, Chen S, Irwin RW, Iwamoto S, Brinton RD. Estrogen protects neuronal cells from amyloid beta-induced apoptosis via regulation of mitochondrial proteins and function. *BMC Neurosci.* 2006; 7:74. [PubMed: 17083736]

- Nilsen J, Irwin RW, Gallaher TK, Brinton RD. Estradiol in vivo regulation of brain mitochondrial proteome. *J Neurosci*. 2007; 27:14069–77. [PubMed: 18094246]
- Rosario ER, Ramsden M, Pike CJ. Progestins inhibit the neuroprotective effects of estrogen in rat hippocampus. *Brain Res*. 2006; 1099:206–10. [PubMed: 16793026]
- Schumacher M, Guennoun R, Stein DG, De Nicola AF. Progesterone: Therapeutic opportunities for neuroprotection and myelin repair. *Pharmacol Ther*. 2007
- Schumacher M, Sitruk-Ware R, De Nicola AF. Progesterone and progestins: neuroprotection and myelin repair. *Curr Opin Pharmacol*. 2008; 8:740–6. [PubMed: 18929681]
- Simpkins JW, Yi KD, Yang SH, Dykens JA. Mitochondrial mechanisms of estrogen neuroprotection. *Biochim Biophys Acta*. 2009
- Singh M. Progesterone-induced neuroprotection. *Endocrine*. 2006; 29:271–4. [PubMed: 16785602]
- Singh M, Dykens JA, Simpkins JW. Novel mechanisms for estrogen-induced neuroprotection. *Exp Biol Med (Maywood)*. 2006; 231:514–21. [PubMed: 16636299]
- Wise PM, Dubal DB, Wilson ME, Rau SW, Bottner M, Rosewell KL. Estradiol is a protective factor in the adult and aging brain: understanding of mechanisms derived from in vivo and in vitro studies. *Brain Res Brain Res Rev*. 2001; 37:313–9. [PubMed: 11744096]
- Woolley CS. Effects of estrogen in the CNS. *Curr Opin Neurobiol*. 1999; 9:349–54. [PubMed: 10395567]
- Yager JD, Chen JQ. Mitochondrial estrogen receptors--new insights into specific functions. *Trends Endocrinol Metab*. 2007; 18:89–91. [PubMed: 17324583]
- Yao J, Irwin RW, Zhao L, Nilsen J, Hamilton RT, Brinton RD. Mitochondrial bioenergetic deficit precedes Alzheimer's pathology in female mouse model of Alzheimer's disease. *Proc Natl Acad Sci U S A*. 2009; 106:14670–5. [PubMed: 19667196]
- Yao J, Hamilton RT, Cadenas E, Brinton RD. Decline in mitochondrial bioenergetics and shift to ketogenic profile in brain during reproductive senescence. *Biochim Biophys Acta*. 2010
- Zhao L, Wu TW, Brinton RD. Estrogen receptor subtypes alpha and beta contribute to neuroprotection and increased Bcl-2 expression in primary hippocampal neurons. *Brain Res*. 2004; 1010:22–34. [PubMed: 15126114]

Research Highlight

- 1.** 17 β -estradiol (E2) protects neurons against specific mitochondrial inhibitors
- 2.** Progesterone (P4) does not protect neurons against mitochondrial inhibitors
- 3.** Co-administration of P4 with E2 abolishes E2 induced neuroprotection
- 4.** Both E2 and P4 potentiates mitochondrial respiration in vitro
- 5.** Co-administration of P4 and E2 results in diminished mitochondrial respiration

Figure 1. Concentration-dependent Response of Mitochondrial Inhibitors

Primary hippocampal neurons were treated with different mitochondrial inhibitors at different concentrations for 24 hours. Cell viability after toxin treatment was measured by Calcein Am fluorescent assay. A, inhibition sites of selected mitochondrial inhibitors; B–F, cell viability assay with treatments of rotenone, 3-NPA, antimycin, KCN, and oligomycin respectively (Bars represent mean cell viability $\pm$ S.E.M., $n=8$ for each group, *, $P<0.05$, individual experiment was repeated 5 times).

Figure 2. Therapeutic Efficacy of E2 Induced Neuroprotection Against Mitochondrial Inhibitors Primary hippocampal neurons were pretreated with increasing concentrations of E2 24 hours prior to exposure to different mitochondrial toxins for another 24 hours. Cell viability after toxin treatment was measured by Calcein Am fluorescent assay. A–E, Therapeutic efficacy of E2 induced neuroprotection against rotenone, 3-NPA, antimycin, KCN, and oligomycin respectively (Bars represent mean therapeutic efficacy $\pm$ S.E.M., $n=8$ for each group, *, $P<0.05$, individual experiment was repeated 5 times).

Figure 3. P4 Exhibited No Neuroprotection Against Mitochondrial Inhibitors

Primary hippocampal neurons were pretreated with increasing concentrations of P4 24 hours prior to exposure to different mitochondrial toxins for another 24 hours. Cell viability after toxin treatment was measured by Calcein Am fluorescent assay. A–E, P4 exhibited no neuroprotection against rotenone, 3-NPA, antimycin, KCN, and oligomycin respectively (Bars represent mean cell viability $\pm$ S.E.M., $n=8$ for each group, individual experiment was repeated 5 times).

Figure 4. P4 abolished E2 induced Neuroprotection Against Mitochondrial Inhibitors

Primary hippocampal neurons were pretreated with E2 (10ng/mL) plus increasing concentrations of P4 (0.1ng/ml – 1000ng/ml) 24 hours prior to exposure to different mitochondrial toxins. Cell viability after 24 hour toxin exposure was measured by Calcein Am fluorescent assay. A&B, P4 abolished E2 induced neuroprotection against 3-NPA and antimycin, respectively (Bars represent mean therapeutic efficacy $\pm$ S.E.M., n=8 for each group, *, P<0.05 compared to inhibitors alone; #, P<0.05 compared to E2 alone + toxin group, individual experiment was repeated 5 times).

Figure 5. E2 and P4 regulation of mitochondrial bioenergetic capacity

Primary hippocampal neurons were treated with E2 alone (10ng/ml), P4 alone (100ng/ml), or E2 (10ng/ml) +P4 (100ng/mL) for 24 hours. Cellular metabolic flux activity was measured using the Seahorse metabolic analyzer. A, Both E2 (Red) and P4 (light blue) increased the maximal mitochondrial respiratory capacity relative to vehicle control (dark blue) ($P < 0.05$ compared to ctrl group (dark blue), $n = 5$ per group), whereas E2+P4 (brown) co-administration had much diminished effect on mitochondrial; B, neither E2 nor P4 or the combination of E2 + P4 affected the anaerobic glycolytic activity as ECAR value remained the same across all treatment groups.